Environmental Decontamination of a Chemical Warfare Simulant Utilizing a Membrane Vesicle-Encapsulated Phosphotriesterase

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Abstract

While technologies for the remediation of chemical contaminants continue to emerge, growing interest in green technologies has led researchers to explore natural catalytic mechanisms derived from microbial species. One such method, enzymatic degradation, offers an alternative to harsh chemical catalysts and resins. Recombinant enzymes, however, are often too labile or show limited activity when challenged with non-ideal environmental conditions that may vary in salinity, pH, or other physical properties. Here, we demonstrate how phosphotriesterase encapsulated in a bacterial outer membrane vesicle (OMV) can be used to degrade the organophosphate chemical warfare agent (CWA) simulant paraoxon in environmental water samples. We also carried out remediation assays on solid surfaces including: glass, painted metal, and fabric that were selected as representative materials which could potentially be contaminated with a CWA.

Introduction

Chemical contamination is a concern for watersheds, aquifers, and soil around the globe. While the nature of the contaminants and properties of the environments are highly variable, limitations in remediation strategies are well conserved: 1) large-scale remediation is limited by the quantity of non-catalytic reagents that can be deployed; 2) chemical catalysts are often costly and must be subsequently removed to limit additional environmental contamination; and 3) biological reagents are often unstable or, in the case of engineered or non-native organisms, cannot be freely released into the environment due to regulatory policies which vary widely between countries.[1-2] To circumvent regulatory concerns associated with engineered organisms, efforts to employ biological reagents for remediation have shifted focus to those enzymes that can be extracted and used outside the confines of the originating cell. [3-4]

Recombinant enzymes have seen significant success in industrial processes, the food industry, and as therapeutics; however, they are often engineered to function under a very well defined narrow set of conditions. [2,5] while microbial bioremediation is heavily explored scientifically, regulatory concerns with dissemination of bacterial species and complications with bacterial viability in harsh environments can prove limiting. [1, 6-8] A variety of bioreactors have been developed and have demonstrated success, however, these processes often require specialized equipment and infrastructure. [9-10] Ideally, bioremediation with isolated recombinant enzymes that could be dispersed at the point of concern would provide the most environmentally friendly process as the enzymes themselves would be resorbed into the environment. Unfortunately, environmental conditions vary greatly in temperature, pH, bacterial composition, and salinity which makes engineering enzymes for environmental use difficult. Also, the enzymes themselves are often degraded via hydrolysis or consumed by resident microbes. Finally, purified recombinant enzymes also have a tendency to lose activity rapidly requiring additives to ensure stability and often requiring an established cold-chain during transport to the point of concern. Despite these limitations, the rapid catalytic activity and the ability of the enzymes to be reabsorbed into the environment make these reagents ideal candidates for field use.

We have previously demonstrated a bacterial outer membrane vesicle (OMV) packaging strategy in which phosphotriesterase (PTE) was encapsulated within an OMV (OMV-PTE) as a novel means for enzyme based remediation of paraoxon (**Figure 1**). [11-12] The OMV-PTE reagent demonstrated improved stability compared to non-encapsulated enzyme (Free-PTE) and remained active through multiple freeze-thaw cycles, lyophilization, and extended exposure to elevated temperature (37 °C). Here, we expand beyond our initial efforts and demonstrate the enzymatic degradation of paraoxon from environmental water samples and on solid surfaces using the OMV-encapsulated PTE. This study details how OMV-encapsulated enzyme maintains activity in real world environments and can potentially be implemented to decontaminate both environmental water sources and solid surfaces such as glass, fabric, and painted metal surfaces.

Materials and Methods

Materials. Restriction enzymes and bacterial strains used for OMV production were obtained from New England Biolabs (Ipswich, MA). Bacterial culture media (Terrific broth), chloramphenicol, ampicillin, and colorpHast (pH 5-10) pH strips by EM-Reagents were obtained from Fisher Scientific (Hampton, NH). The PESTANAL analytical standard, paraoxon-ethyl, was purchased from Sigma-Aldrich (St. Louis, MO). All other buffers and salts were purchased from either Sigma Aldrich or Fisher Scientific.

Construction, Expression, and Purification of PTE Reagents and other Proteins. Cloning, expression, and purification of recombinant PTE and OMV-PTE have been described extensively elsewhere but will be briefly described here. [11-13] The gene encoding phosphotriesterase from *Brevundimonas diminuta* (EC 3.1.8.1) was synthesized by Genscript (Piscataway, NJ) and cloned to the bacterial expression vector pET28b for expression and purification. Directed encapsulation of PTE in bacterial OMVs is accomplished using a two plasmid system; one that encodes a modified version of PTE and a second plasmid encoding a recombinant membrane protein. [11-12] In this instance, the anchor protein is a truncated version of the outer membrane porin protein OmpA which presents a small peptide tag, SpyTag, at its N-terminus which resides in the periplasmic space. To facilitate packaging, PTE is expressed with a C-terminal fusion termed SpyCatcher which will spontaneously form an isopeptide bond with the SpyTag peptide. The SpyCatcher/SpyTag system in described extensively elsewhere. [14-15]

Expression of the anchor protein and the PTE enzyme were controlled by two inducible promoters (PTE – arabinose, OmpA – lactose) which were activated separately at different time points during culture growth. Expression continued for a minimum of 16 hours following induction of both constructs. Successive centrifugation cycles were used to remove cells from the culture media. After each cycle, culture media (supernatant) was decanted to a sterile, 500 mL centrifugation bottle. A final filtration step using a 0.45 micron filter was employed to ensure the removal of any residual bacteria and large protein aggregates from the culture media. OMVs were pelleted from 35 mL aliquots of culture media via ultracentrifugation at 129,000 x g. The OMV-PTE pellet was resuspended in 150 mM N-Cyclohexyl-2-aminoethanesulfonic acid (CHES (pH 8.0)), quantitated via NanoSight, aliquoted to microfuge tubes, and snap frozen in a methanol dry ice bath. A fraction of these samples were stored at -80°C while the remainder

were lyophilized using a VirTis Benchtop K Freeze Dryer (SP Industries, Warminster, PA). Individual samples were removed and inspected for water content.

The protocols for the cloning, expression, and purification of PTE are extensively described elsewhere. [13, 16] Expression of enzyme was induced with β-D-thiogalactopyranoside and continue for a minimum of 12 hours at 30□C. Cell pellets were stored at -80□C for a minimum of 3 hours to enhance cell lysis. Cells were lysed via sonication followed by separation of soluble material from insoluble cellular debris via centrifugation. Soluble material was batch incubated with nickel nitrilotriacetic acid coupled resin and eluted via imidazole. Eluted enzyme was further purified via FPLC then snap frozen in CHES buffer (pH 8.0) containing 25% glycerol at a final concentration of 15 uM. This material was used as a control where indicated, specifically in the water decontamination experiments. For other comparative studies, enzyme was diluted 1000-fold in CHES buffer (pH 8.0) and frozen prior to lyophilization as described above. This sample will be referred to as "Free-PTE" and can be found in the *Supplemental Information Figure S1*.

Water Samples. Environmental water samples were collected directly into 50 mL tubes at the designated locations and stored at 4 °C. The collection occurred during a medium intensity rain event in Washington, DC in November 2016. No purification, filtration, or centrifugation was performed on the samples prior to utilization in the remediation assays. Their pH was determined via pH strip. Details for sample collection and images of each location can be found in Figure 2 and *Supplemental Information*. Artificial seawater was prepared as a modified recipe of the one initially described by Schubbe *et al*. [17] The modified solution is described in Zheng *et al.* and can be found in the *Supplemental Information*. [18]

PTE Kinetic Assays. Assays were conducted in either 50 mM CHES buffer (pH 8.0) or directly in the designated water sample. All assays were performed at 25 °C using the PESTANAL paraoxon standard diluted 1000-fold in either buffer or environmental sample. Following addition of the paraoxon, environmental water samples were aged approximately 30 minutes before addition of Free-PTE or OMV-PTE. Enzyme-mediated hydrolysis of the paraoxon substrate to *p*-nitrophenol was monitored at 405 nm and 348 nm (*p*-nitrophenol isosbestic point). Initial velocities were determined by the slope of the first 5 min of reaction and were utilized to compare the relative quantity of active PTE in each environmental water sample normalized to control activity in CHES buffer at the same PTE concentration and can be found in the *Supplemental Information Figure S2*. In all experiments control reactions consisting of enzyme only, substrate only, and OMV lacking PTE were included. All data represents means (±SD) of triplicate experiments. Unless otherwise indicated, the control enzyme (Free-PTE) was prepared and snap-frozen in 25% glycerol to ensure activity while the OMV-PTE samples were frozen in buffer and then lyophilized.

Vesicle Rupture pH Sensitivity. To determine whether or not the internal OMV microenvironment resists variations to solution pH, OMV encapsulated pH sensitive pHluorin proteins were utilized. [19] Fluorescence was monitored (ex 380 nm, em 510 nm) in the presence and absence of 1% Triton X-100 and at pH values of 5.5 and 7.5. The use of 1% Triton X-100 to rupture the OMV was verified via NanoSight particle tracking.

NanoSight. Control and OMV-PTE sample quantitation was performed on a NanoSight LM10 system (Salisbury, UK) using NTA 2.3 Nanoparticle Tracking and Analysis software. Camera shutter (13.8 ms) and gain (324) were manually optimized to enhance data collection. Videos (90 s) were taken, and frame sequences were analyzed under auto particle detection and tracking parameters. All samples were run at RT and allowed to equilibrate prior to analysis. For both control and enzyme-filled OMVs, average OMV concentration was approximately $1x10^{12}$ particles/mL. Samples with higher concentrations were diluted to ensure consistency between control OMV samples and OMV-PTE samples.

Vesicle rupture was verified in the presence of Triton X-100 by the absence of particles at increasing Triton X-100 percentages. Gold nanoparticles were utilized as a positive control to verify particle tracking was not inhibited by the presence of up to 5% Triton X-100 and can be found in the *Supplemental Information Figure S3*.

Surface Remediation. For evaluation of OMV performance in decontamination of surfaces, target exposures utilized the standard challenge level of 10 g/m^2. [20] The coupons were 10.1 cm^2 comprised of MultiCam 50/50 nylon/cotton Army Combat Uniform (ACU) fabric, polyurethane paint coated aluminum coupons, and borosilicate glass. The 10 g/m^2 target challenge was applied to the surfaces as two equally sized droplets of 4 µL each and aged 1 h prior to processing. As a control, coupons of this type were placed directly into isopropanol (15 mL) for 30 min to assess maximal paraoxon extraction. A second control used a rinse step, a low pressure stream of 15 mL aqueous soap solution (0.59 g/L Alconox), following aging and a 10 s soak in the rinse solution prior to soaking in isopropanol (15 mL). This is the typical protocol for evaluation of agent retention on surfaces. [20] The final set of conditions evaluated decontamination by the OMV-PTE solution. OMV-PTE (2 mL solution, resuspended from lyophilized material to ~$1x10^{12}$ OMV/mL) was applied to the aged coupon, as indicated above, and incubated for 30 min. The coupon was rinsed with water or CHES buffer (15 mL) followed by a 30 min isopropanol extraction (15 mL). In all cases, the paraoxon content of the isopropanol extract was evaluated. The aqueous rinsate was also sampled for immediate analysis of paraoxon conversion and at later time points as indicated in the text.

Paraoxon HPLC Analysis. For paraoxon analysis, a Shimadzu High Performance Liquid Chromatography (HPLC) system with dual-plunger parallel flow solvent delivery modules (LC-20AD) and an auto-sampler (SIL-20AC; 40 µL injection volume) coupled to a photodiode array detector (SPD-M20A; 277 nm) was used. The stationary phase was a C18 analytical column (Luna, 150 mm x 4.6 mm, 3 µm diameter; Phenomenex, Torrance, CA) with an isocratic 45:55 acetonitrile: 1% aqueous acetic acid mobile phase (1.2 mL/min). [21] All data represents means (±SD) of triplicate experiments.

Results and Discussion

Environmental remediation of chemical contaminants is a complex problem as no two environmental samples are the same. Of significant concern for any reagent produced for field deployment is the reliability/activity of this material once removed from the laboratory and challenged with real-world environmental conditions. Below we describe our efforts to

demonstrate and characterize the activity of OMV-encapsulated PTE in a number of different environmentally sourced water samples and solid surface decontamination.

Water samples

In an effort to select a variety of challenges that a remediation reagent would likely be exposed to in real world situations, samples were manually collected from the following locations during a medium rain fall: 1) stagnant water in a dirt puddle (Dirt Puddle), 2) metal roof building downspout (Downspout), 3) flowing water runoff prior to entering underground drainage pipes (Drain), 4) stagnant water in a paved parking lot (Pavement Puddle), and from a 5) Pond (**Figure 2**). The pH of each sample was taken via pH strip with all samples falling between pH 5 and 6 (data not shown).

Buffered Environmental Remediation. Enzymatic activity is often sensitive to pH fluctuations which is an easily controlled consideration for laboratory based remediation applications. Initial evaluations in environmental samples were completed with the addition of CHES buffer to eliminate pH as a variable. In all cases, lyophilized OMV-PTE was used. Lyophilization is expected to be the preferred method of stabilization for long term reagent storage and transportation as it does not require a cold chain and removal of water significantly reduces reagent weight allowing for greater quantities of active agent to be transported. As previously demonstrated, unprotected PTE can exhibit reduced stability post-lyophilization and therefore, Free-PTE that was purified and then snap frozen and stored at -80°C was utilized instead of lyophilized PTE for all environmental remediation comparisons (**Supplemental Figure S1**). [12]

Environmental samples were buffered to pH 8, and kinetic assays were carried out in 96-well microtiter plates using paraoxon as a substrate while tracking *p*-nitrophenol formation via absorbance at 348 nm, the isosbestic point. The sample composition for remediation in buffered environmental samples was 35% CHES buffer and 65% environmentally sourced water at a 100 µL total volume. Following a brief incubation, Free-PTE or rehydrated, lyophilized OMV-PTE was added directly to the sample. Percent PTE activity was determined by initial velocity measurement, slope over 5 min, and normalized to PTE activity based on matching PTE concentration in control paraoxon conversion assays performed in CHES buffer alone (**Figure 3 & Supplemental Figure S2**). To ensure that residual CHES salts present in the lyophilized OMV-PTE samples did not affect enzyme activity, decreasing volumes of the stock concentrations as defined in the Materials and Methods section were assayed in parallel (**Supplemental Figure S3**).

The lyophilized OMV-PTE performed at or better than the fresh Free-PTE in environmental water sources and across nearly all PTE dilutions. The largest deviation was observed in the Dirt Puddle remediation samples in which OMV-PTE outperformed Free-PTE by 48 ± 9% activity on average. The Dirt Puddle sample contained the most solid material and observable particulates; the physical barrier provided by the OMV likely contributed to this performance enhancement. It is also important to note that the percent enzyme activity across all PTE dilutions is consistent indicating that there are no significant concentration dependent factors in the buffered environmental remediation assay. Compared to control reactions using

optimized enzyme conditions, the percent activities for environmental samples are also very high with 100.0 ± 9.5% and 79.1 ± 17.9% activity for OMV-PTE and Free-PTE, respectively. As previously mentioned, free enzyme controls were performed with enzyme stored at -80°C in the presence of a cryoprotectant. Due to inactivation of the Free-PTE enzyme from the lyophilization process these assays were not performed with lyophilized Free-PTE which would result in significantly reduced activity across all samples.

Unbuffered Environmental Remediation. Since it is unlikely that environmental remediation will afford the opportunity to buffer an entire contaminated area, paraoxon remediation in unbuffered environmental water sources was also evaluated to determine if reaction kinetics would be maintained. Testing in unbuffered environmental water sources produced an overall reduction in the average percent enzyme activity to 72.9 ± 7.7% for lyophilized OMV-PTE and 77.8 ± 22.8% for fresh Free-PTE that can be attributed primarily to a reduction in assay pH. Though Free-PTE does show a slightly higher percent enzyme activity in these assays it is important to remember that the Free-PTE sample has not been lyophilized which has been shown to dramatically reduce free enzyme activity. Additionally, while the activities between Free-PTE and lyophilized OMV-PTE are very similar in unbuffered water samples the lyophilized OMV-PTE was much more consistent across samples and has the added logistical benefits of reduced reagent weight and easier storage and transport to the point of contamination. Comparing the PTE dilutions within each contaminated water source reveals something interesting. For the Free-PTE there is a clear trend toward reduced activity at lower PTE concentrations that is not observed in the OMV-PTE dilutions (**Figure 4**). While these are unbuffered remediation assays carried out in 95-99% environmental water, there is some CHES buffer present in the OMV-PTE and Free-PTE, representing a final concentration of: 2.5, 2, 1.5, 1, and 0.5 mM CHES for the 5-1 μL PTE dilutions, respectively. None of these concentrations resulted in an appreciable increase in pH; however, it may have contributed to the observed decrease in activity of the Free-PTE at lower PTE and CHES concentrations. Reduced PTE activity in solution with a pH below pH 7.5 has been described in the literature and is consistent with the observations described here. [22]

As with the previous assays, lyophilized OMV-PTE outperformed the Free-PTE in the Dirt Puddle assay at all dilutions as well as at all of the most dilute PTE levels across all of the samples (**Figure 4**). The most dilute PTE, 1 μL in each category, models the most realistic representation of true environmental remediation in that the remediation reagent contributes little-to-no buffer concentration to the environment, 99% environmentally sourced water, and remediation is carried out at very dilute enzyme concentrations. Previously, we demonstrated that encapsulating PTE within the OMV greatly extended the length of time for which it remained active under elevated assay temperatures and through multiple freeze-thaw cycles. [11-12] This offers potential for use of the OMV-PTE as a remediation reagent with a long active residence time once added to the environmental point of concern. Additionally, over time the OMV-PTE would be naturally resorbed by the environment, eliminating the need for additional site remediation to remove the catalyst, in this can OMV-PTE, itself.

OMV Resistance to pH Change. In the previous assays, it was observed that the activity of OMV-PTE was not significantly affected by the pH range that was inherent in the environmental

samples. We hypothesized that in some proportion of the OMV-PTE population, membrane and periplasmic proteins would be present and functional to some level conveying protection from environmental conditions such as pH and salinity. To test this theory, a pH sensitive fluorescent protein, pHluorin, was cloned into a bacterial expression vector modeled after a construct that facilitates addition of an N-terminal lipoylation tag as described by Stathopoulos *et al.*[23] The construct used here lacks the membrane spanning domains and therefore anchors the pHluorin protein directly to the inner surface of the outer membrane. As with the OmpA anchor used for PTE packaging, active anchoring to the outer membrane facilitates efficient loading of pHluorin into nascent OMV.

PHluorin is a unique protein whose fluorescence intensity decreases as pH decreases. To monitor the ability of OMV to protect cargo from pH extremes, purified OMV-pHluorin were subjected to buffered solutions of pH 7.5 and 5.5 (**Figure 5**). Fluorescence was measured for 60 minutes during which there was a small reduction in fluorescence intensity (13%) observed between the two slightly acidic solutions. To verify the role of the OMV in affording this protection, OMV-pHluorin samples containing 1% Triton X-100 were monitored in parallel. As has been previously described, 1% Triton X-100 is sufficient to rupture OMVs and release encapsulated proteins (**Supplemental Figure S4 & S5**). [11] Here we show that in the presence of the detergent, fluorescent intensity for the pH 5.5 solution is decreased significantly with a resulting 45% loss of fluorescence intensity. No reduction in pHluorin fluorescence was observed through exposure of the pHluorin protein to Triton X-100 itself. This assay demonstrates that the OMV encapsulated pHluorin did not experience the full pH dependent fluorescence reduction at pH 5.5 compared to the ruptured OMV samples. The mechanism by which the OMV resists internal pH changes or how the internal OMV microenvironment protects the pHluorin is not well understood; however, the diverse transmembrane porin proteins present in the OMV may contribute to ion selectivity entering or leaving the OMV.

Combined with the results of the buffered and unbuffered enzyme assays, the pHourin experiments clearly show that OMV encapsulation affords some pH stabilizing effect to cargo proteins. Interestingly, Su *et al.* reported protection to PTE when enzyme was localized to the exterior of OMVs. [24] As with the results shown here OMV-associated enzyme had higher percent activity at lower pH when compared to a free enzyme. Clearly additional studies are necessary to determine what roles OMVs are playing in stabilizing reaction conditions.

High Salt Enzyme Assays. Environmental remediation is not just limited to fresh water contamination. Therefore, determining activity for the Free-PTE and lyophilized OMV-PTE at increasing salinity levels and in a seawater simulant is also of interest. To test for salinity effects on enzyme activity all samples were maintained at pH 8.0 with 50 mM CHES buffer and concentrated NaCl was added to produce salinities up to 1,770 mM or 9.9% (**Figure 6**). OMV-PTE samples maintained a higher level of enzyme activity at increasing NaCl concentrations dropping to 39% retained activity at 1,770 mM NaCl. This is compared to no observable activity of the Free-PTE. Seawater simulant (~3.5% salinity) was used in place of the environmentally sourced water to evaluate performance at decreasing PTE concentrations in both buffered and unbuffered samples. When comparing the percent retained activity of the buffered and

unbuffered paraoxon contaminated seawater simulant, the OMV-PTE was minimally impacted at a 2.6 ± 5.8% reduction in activity in unbuffered seawater compared to a 27.7 ± 5.5% reduction in activity for the unbuffered Free-PTE (**Supplemental Figure S6**). The absolute enzyme activity of both OMV-PTE and Free-PTE compared to the CHES buffer controlled hydrolysis of paraoxon was consistent with the activity reduction based on salinity effects observed in **Figure 6**.

Surface Remediation. Beyond remediation of environmental sites, decontamination of critical equipment and personnel using the OMV-PTE reagent is also of interest. Surface decontamination is applicable to protection of personnel as well as treatment of equipment, with utility extending to civilian, agricultural, and Department of Defense considerations. Frequently, surface decontamination is achieved through a protocol designed to dislodge the contaminant from the surface with minimal regard to subsequent residence or accumulation in the soil. Here we demonstrate that solutions of rehydrated OMV-PTE can be used both to remove significant concentrations of paraoxon from the surface and completely degrade the compound in wash solutions mitigating subsequent environmental contamination.

Utilizing the OMV-PTE remediation reagent, we evaluated paraoxon removal in both unbuffered and buffered surface remediation assays on materials chosen to represent relevant surfaces such as painted metals, textiles, and glass. When the painted coupons are contaminated with paraoxon, aged, and extracted using isopropanol, 9.75 g/m^2 of the 10 g/m^2 paraoxon is recoverable (**Figure 7**). When coupons are rinsed with deionized water or buffered solution prior to extraction 11% of the paraoxon contaminant is removed from the surface as indicated by the content of the isopropanol extract (8.60 g/m^2). Utilizing OMV-PTE in an unbuffered surface remediation assay resulted in an initial paraoxon removal of 46% with an additional 10% paraoxon decontamination occurring in the rinsate over a period of 1.5 h following extraction. This lower rate of conversion was expected due to performing the remediation assay in the absence of buffer.

A subsequent series of experiments were performed using CHES buffered OMV-PTE and a similarly buffered rinse solution. While buffer did not improve removal of paraoxon from the coupon surfaces in the absence of OMV-PTE, it did improve conversion of paraoxon both on the surface and in the rinsate. When buffered OMV-PTE was utilized, the retained paraoxon was 2.19 g/m^2 (76% removed) compared to 5.25 g/m^2 for the unbuffered rinsed samples. In addition, the paraoxon content of the rinsate was below the limits of detection for the HPLC method within 1 h of extraction demonstrating ongoing enzymatic catalysis in the rinsate with rates significantly higher than those observed in water. Improved surface decontamination may be attainable via a two-step process in which an optimized surface extraction solution is initially used followed by a solution based enzymatic degradation step.

Similar evaluations were completed for glass and fabric surfaces (**Figure 7**). Glass surfaces retain less paraoxon under simple rinse conditions (3.4 g/m^2) than that noted for the painted surface. When buffered OMV-PTE was used on this surface, little to no paraoxon was detected in the isopropanol extract. This difference, as compared to painted surfaces, is likely a reflection of the penetration of paraoxon into the texture of the paint and poor penetration of the

OMV solution into these spaces. As with the painted metal samples, the rapid catalysis of the paraoxon by OMV encapsulated PTE ensured little to no paraoxon was detectable in the rinsate for these surfaces immediately following collection.

The representative fabric surface retains significantly more paraoxon than either the glass or painted surfaces examined when treated with the OMV-PTE solution. Similar to the painted metal surface, the porosity of the fabric likely contributes to the retention of the paraoxon and difficulty in decontamination through simple washing. Paraoxon that was displaced from the fabric sample was rapidly degraded and no target was detected in the rinsate solution immediately following collection. While beyond the scope of these initial studies, prolonged incubation with OMV-PTE solutions allows for degradation of the paraoxon within the fabric and will be surveyed in future studies.

Environmental Implications

When developing a remediation reagent it is important to consider the environmental impact of its production, transportation to the point of concern, the potential toxicity of the remediation reagent itself, and any additional steps necessary to remove residual catalysts or other reaction products. The bacterial OMV offers a unique tool for environmental remediation in that bacterial cultures are easily scalable to meet the large quantity needs when considering the demands of treating something on the order of a contaminated pond, field, stream, or facility. The OMV-PTE reagent itself can be lyophilized which significantly simplifies packaging and distribution to the point of concern. Additionally, lyophilized OMV-PTE reagents remain active when stored at room temperature suggesting that these materials could potentially be stockpiled and simply rehydrated when need arises. Also, since the bacterially derived OMV themselves are not bacteria and are incapable of performing cellular functions or replicating this eliminates any concerns associated with releasing an active mutated bacteria into the wild. As fully biological and biodegradable reagents, there is also little concern for releasing large quantities of these reagents into natural environments as the biomolecules would be consumed by native microbial flora. While this is true for the OMVs, secondary products of bioremediation is also a concern. In the example given here paraoxon is hydrolyzed to *p*-nitrophenol which is less toxic but still harmful to the environment when accumulated or persistent. Other organophosphate compounds, particularly chemical warfare agents, are often degraded to products that are nearly as toxic as the original substrate. Ideally, multiple enzymes could be combined to continue the degradation process, targeting products from previous enzymatic steps to lead to complete neutralization of the compound. We anticipate that such enzyme cascades could be assembled either in a single host bacterium or through a scaffold catalytic system similar to those described in the literature for the degradation of cellulose biomass.[25]

While this manuscript details the enzymatic break down of paraoxon, a pesticide analog for organophosphate-based chemical warfare agents, by PTE a variety of organophosphates are also used in agriculture leading to accumulation of these compounds in waterways and soils. While PTE may not be capable of digesting all organophosphates this packaging and delivery technique represents a model system that can be adapted with alternate enzymes, varying in target specificity, with the potential to develop mixtures of enzymes to create broad spectrum

OMV based remediation reagents. We envision the utilization of OMV packaging strategies to develop enzyme-mediated bioremediation strategies capable of eliminating/neutralizing a broad range of chemical compounds including: nitroenergetics, polyphenols, or other contaminants of concern.

Finally, as shown here, OMV-encapsulation has the potential to significantly improve the distributability of enzymes into real-world environments. While here we describe of efforts at bioremediation, PTE and other enzymes have also shown significant promise as environmental sensors. In a recent publication we demonstrated that PTE could be integrated into an electrochemical detector and used for environmental monitoring for organophosphate compounds. [26] Currently we are striving to combine these two research areas to further exploit the protective nature of OMVs with the hopes of developing long-lasting environmental sensors.

Supporting Information

The Supporting Information is included as an additional attachment.

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Notes

The authors declare no competing financial interest.

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References

1. Karigar, C. S.; Rao, S. S., Role of microbial enzymes in the bioremediation of pollutants: a review. *Enzyme Res* **2011,** *2011*, 805187.
2. Singh, R.; Kumar, M.; Mittal, A.; Mehta, P. K., Microbial enzymes: industrial progress in 21st century. *3 Biotech* **2016,** *6*, 174.
3. Dec, J.; Bollag, J.-M., Use of Enzymes in Bioremediation. In *Pesticide Biotransformation in Plants and Microorganisms*, ACS Symposium Series: 2000; pp 182-193.
4. Peixoto, R. S.; Vermelho, A. B.; Rosado, A. S., Petroleum-degrading enzymes: bioremediation and new prospects. *Enzyme Res* **2011,** *2011*, 475193.

5. Jegannathan, K. R.; Nielsen, P. H., Environmental assessment of enzyme use in industrial production – a literature review. *J Clean Prod* **2013,** *42*, 228-240.
6. Pushpanathan, M.; Jayashree, S.; Gunasekaran, P.; Rajendhran, J., Microbial Bioremediation: A Metagenomic Approach. In *Mirobial Biodegradation and Bioremediation*, Das, S., Ed. Elsevier: 2014; pp 407-419.
7. Urgun-Demirtas, M.; Stark, B.; Pagilla, K., Use of genetically engineered microorganisms (GEMs) for the bioremediation of contaminants. *Crit Rev Biotechnol* **2006,** *26*, 145-64.
8. Singh, J. S.; Abhilash, P. C.; Singh, H. B.; Singh, R. P.; Singh, D. P., Genetically engineered bacteria: an emerging tool for environmental remediation and future research perspectives. *Gene* **2011,** *480*, 1-9.
9. Robles-Gonzalez, I. V.; Fava, F.; Poggi-Varaldo, H. M., A review on slurry bioreactors for bioremediation of soils and sediments. *Microb Cell Fact* **2008,** *7*, 5.
10. Nath, A.; Chakraborty, S.; Bhattacharjee, C., Bioreactor and Enzymatic Reactions in Bioremediation. In *Microbial Biodegradation and Bioremediation*, Das, S., Ed. Elsevier: 2014; pp 455-495.
11. Alves, N. J.; Turner, K. B.; Daniele, M. A.; Oh, E.; Medintz, I. L.; Walper, S. A., Bacterial nanobioreactors-directing enzyme packaging into bacterial outer membrane vesicles. *ACS Appl Mater Interfaces* **2015,** *7*, 24963-72.
12. Alves, N. J.; Turner, K. B.; Medintz, I. L.; Walper, S. A., Protecting enzymatic function through directed packaging into bacterial outer membrane vesicles. *Sci Rep* **2016,** *6*, 24866.
13. Breger, J. C.; Ancona, M. G.; Walper, S. A.; Oh, E.; Susumu, K.; Stewart, M. H.; Deschamps, J. R.; Medintz, I. L., Understanding how nanoparticle attachment enhances phosphotriesterase kinetic efficiency. *ACS Nano* **2015,** *9*, 8491-8503.
14. Li, L.; Fierer, J. O.; Rapoport, T. A.; Howarth, M., Structural analysis and optimization of the covalent association between SpyCatcher and a peptide tag. *J Mol Biol* **2014,** *426*, 309-317.
15. Zakeri, B.; Fierer, J. O.; Celik, E.; Chittock, E. C.; Schwarz-Linek, U.; Moy, V. T.; Howarth, M., Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesion. *PNAS* **2012,** *109*, e690-697.
16. Breger, J. C.; Walper, S. A.; Oh, E.; Susumu, K.; Stewart, M. H.; Deschamps, J. R.; Medintz, I. L., Quantum dot display enhances activity of a phosphotriesterase trimer. *Chem Commun (Camb)* **2015,** *51*, 6403-6.
17. Schubbe, S.; Williams, T. J.; Xie, G.; Kiss, H. E.; Brettin, T. S.; Martinez, D.; Ross, C. A.; Schuler, D.; Cox, B. L.; Nealson, K. H.; Bazlinski, D. A., Complete genome sequence of the chemolithoautotrophic marine magnetotactic coccus strain MC-1. *Appl Environ Microbiol* **2009,** *75*, 4835-4852.
18. Wang, Z.; Leary, D. H.; Malanoski, A. P.; Li, R. W.; Hervey, W. J. t.; Eddie, B. J.; Tender, G. S.; Yanosky, S. G.; Vora, G. J.; Tender, L. M.; Lin, B.; Strycharz-Glaven, S. M., A previously uncharacterized, nonphotosynthetic member of the Chromatiaceae is the primary CO2-fixing constituent in a self-regenerating biocathode. *Appl Environ Microbiol* **2015,** *81*, 699-712.
19. Mahon, M. J., pHluorin2: an enhanced, ratiometric, pH-sensitive green florescent protein. *Adv Biosci Biotechnol* **2011,** *2*, 132-137.

20. Lalain, T.; Mantooth, B.; Shue, M.; Pusey, S.; D., W. *Chemical contaminant and decontaminant test methodology source document*; Edgewood Chemical Biological Center: 2012.
21. Genreegzi, Y. T.; Foster, G. D.; Khan, S. U., Simultaneous determination of carbaryl, malatihion, fenitrothion, and diazinon residues in sesame seeds (Sesamum indicum L.). *J Agric Food Chem* **2000,** *8*, 5165 - 5168.
22. Cheng, Y.; Karns, J. T., A, Characterization of a phosphotriesterase from genetically-engineered *Escherichia coli*. *J Environ Sci Health B* **1998,** *33*, 347 - 367.
23. Stathopoulos, C.; Georgiou, G.; Earhart, C. F., Characterizaation of *Escherichia coli* expressing an Lpp' OmpA(46-159)- PhoA fusion protein localized in the outer membrane. *Appl Environ Microbiol* **1996,** *45*, 112-119.
24. Su, F.-H.; Tabanag, I. D.; Wu, C.-Y.; Tsai, S.-L., Decorating outer membrane vesicles with organophosphorus hydrolase and cellulose binding domain for organophosphate pesticide degradation. *Chemical Engineering Journal* **2017,** *308,* 1-7.
25. Park, M.; Sun, Q.; Liu, F.; DeLisa, M. P.; Chen, W., Positional assembly of enzymes on bacterial outer membrane vesicles for cascade reactions. *PloS One* **2014**, 9, e97103.
26. Hondred, J. A.; Breger, J. C.; Alves, N. J.; Trammell, S. A.; Walper, S. A.; Medintz, I. L.; Claussen, J.C., Printed graphene electrochemical biosensors fabricated by inkjet maskless lithography for rapid and sensitive detection of organophosphates. *ACS Appl Mater Interfaces* **2018**, 10, 11125-11134.

Figures

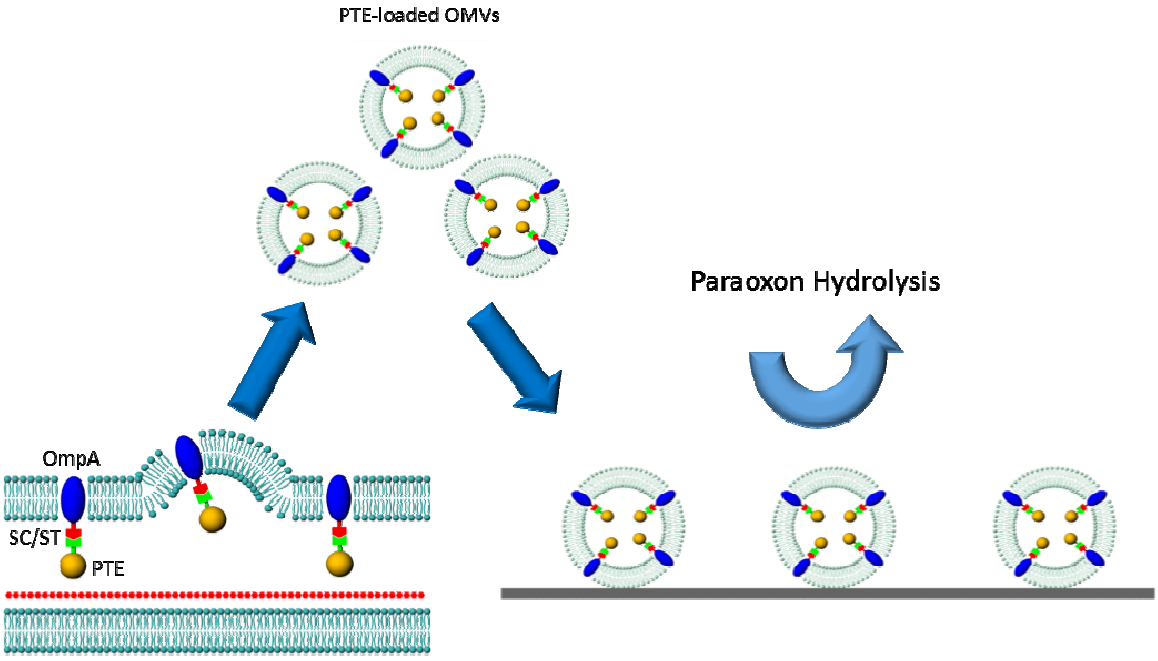

Figure 1: Packing phosphotriesterase into bacterial OMV

The PTE enzyme was expressed with a C-terminal fusion (SpyCatcher) and localized to the periplasmic space where an engineered porin protein (OmpA) presenting a complementary peptide tag (SpyTag) was inserted into the outermost membrane. Formation of an isopeptide bond between SpyCatcher/SpyTag anchored PTE to the membrane. As OMVs are formed, PTE is pulled into the nascent particle ultimately resting in the lumenal cavity. Enzyme-filled OMVs are purified from the culture media and stored under a range of conditions. The OMV-PTE reagents can then be utilized to hydrolyze a range of organophosphate compounds.

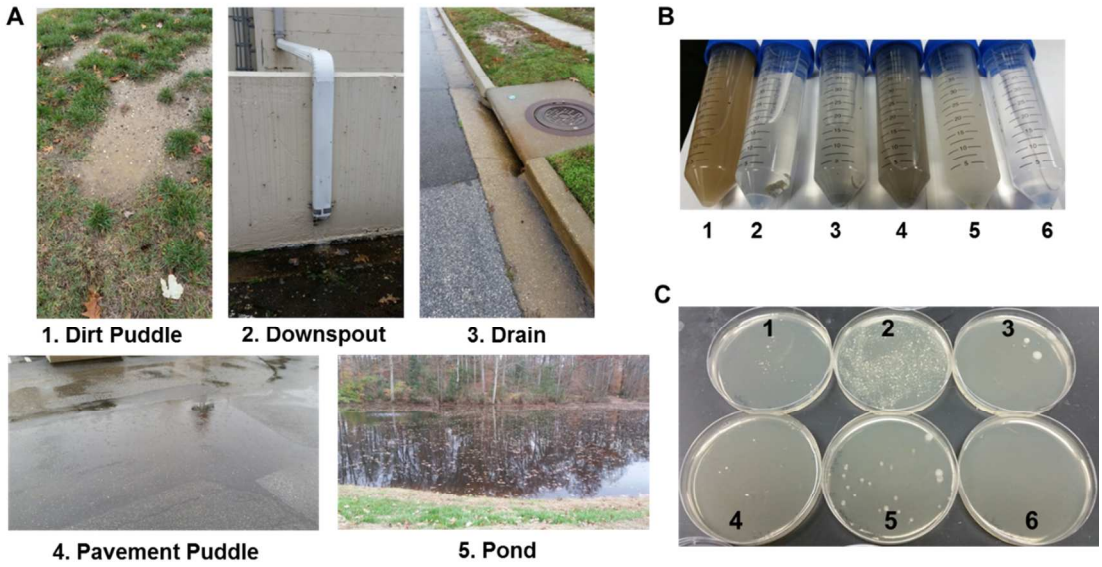

Figure 2: A. Pictures of the environmental water sources collected. B. Samples from each source did not undergo any centrifugation or purification steps prior to implementation in paraoxon remediation assays. C. All samples demonstrated diverse bacterial growth, with the negative control (Sample 6) showing no growth.

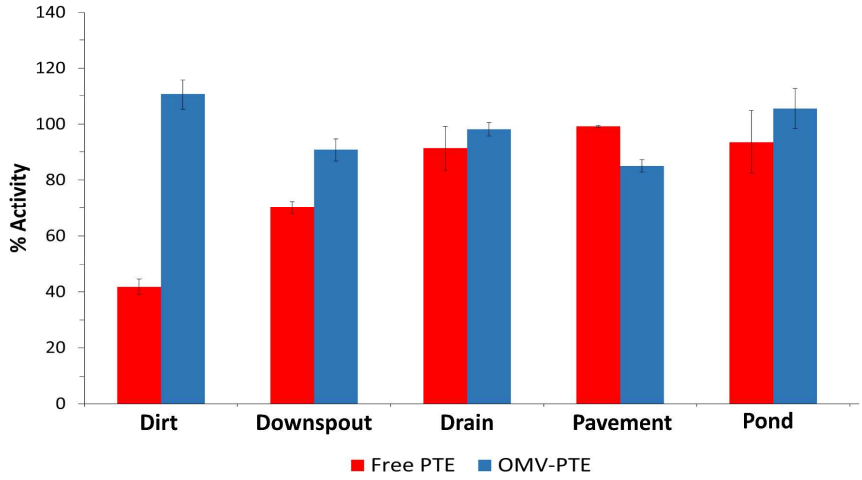

Figure 3: Remediation comparison of lyophilized OMV encapsulated PTE (OMV-PTE) to Free-PTE in CHES buffered environmental samples. The environmental samples were buffered to pH 8 with CHES and initial velocity (348 nm) determined to compare rates of substrate hydrolysis. Percent enzyme activity was normalized to Free-PTE activity in CHES buffered controls (*Supporting Information*). In nearly all cases, lyophilized OMV-PTE performed the same or better than Free-PTE with the largest improvement observed in the Dirt Puddle sample. All data represents means (±SD) of triplicate experiments.

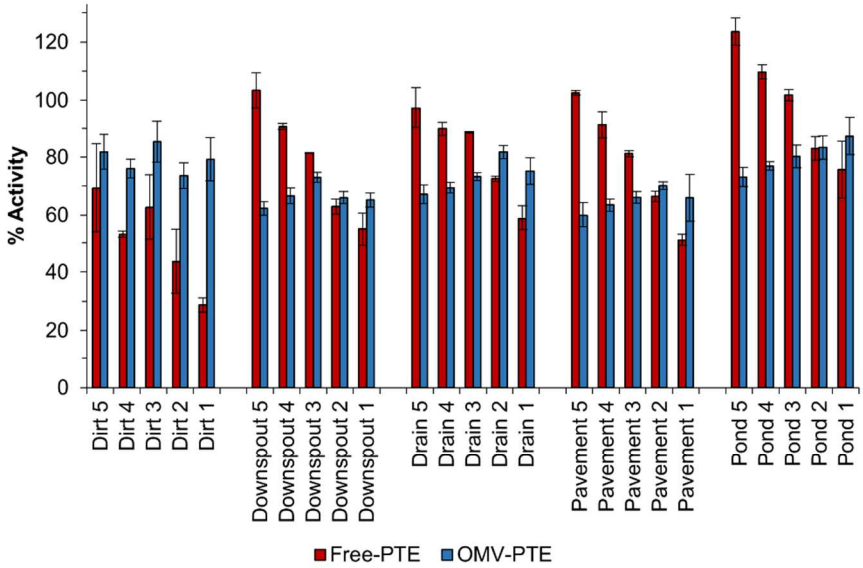

Figure 4: Remediation comparison of lyophilized OMV-PTE to fresh Free-PTE in unbuffered environmental samples. Initial velocity (348 nm) determination was carried out at decreasing PTE concentrations and then percent enzyme activity was normalized to CHES buffered controls. The bars in the "1" categories (99% environment sample) represent the most realistic environmental remediation condition and demonstrate that lyophilized OMV-PTE performs better than Fresh-PTE in all cases with the biggest improvement observed in the Dirt Puddle. All data represents means (±SD) of triplicate experiments.

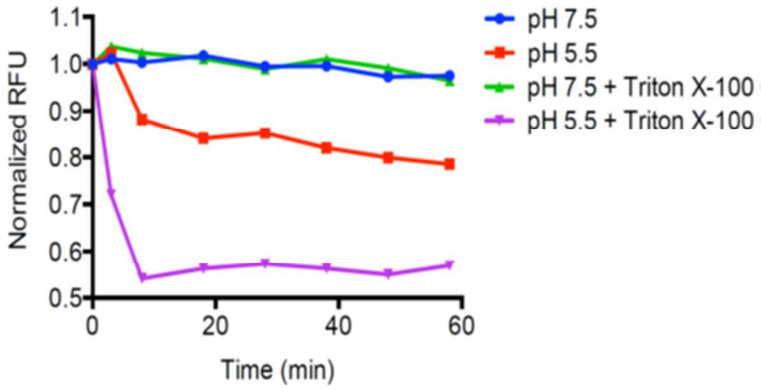

Figure 5: OMV encapsulated pH sensitive pHluorin proteins were utilized to probe the intra-OMV microenvironment at pH 7.5 and 5.5. Triton X-100 was utilized to rupture the OMV and expose the pHluorin to solution pH. This demonstrates that the OMV resists intra-pH changes by maintaining a more optimal enzymatic microenvironment as evidenced by less reduction in encapsulated pHluorin fluorescence.

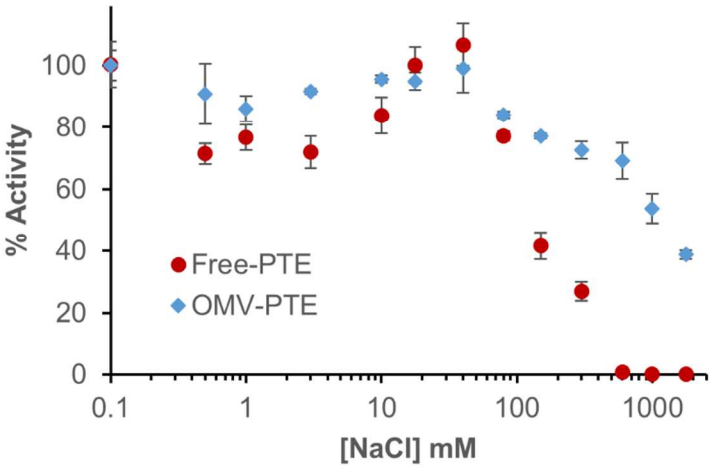

Figure 6: Enzyme activity of OMV-PTE and Free-PTE at increasing NaCl levels in pH 8.0 CHES buffer were assessed to determine potential ionic protection within the OMV microenvironment. The Free-PTE enzyme activity dropped off more rapidly at increasing salinity compared to OMV encapsulated PTE demonstrating some ionic protection afforded by the OMV. All data represents means (±SD) of triplicate experiments.

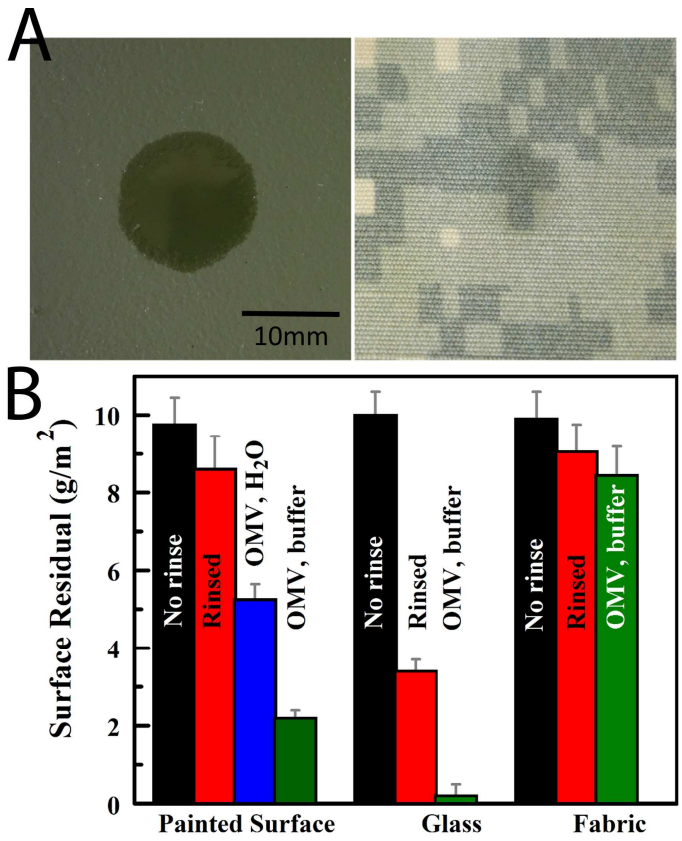

Figure 7: A) Photographs of the painted metal surface and the textile used as representative materials. The surfaces have been exposed to 5µL of paraoxon and aged for 5 min. B) Recovery of paraoxon in isopropanol extracts: isopropanol extract only (black), rinse followed by isopropanol extract (red), treatment with OMV in water followed by rinse and isopropanol extract (blue), treatment with OMV in buffer followed by rinse and isopropanol extract (green).

TOC (3.33” wide by 1.875” tall)